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13. ABSTRACT *(Maximum 200 Words)*

uPAR (urokinase-type plasminogen activator receptor) is a key player in metastasis of breast cancer cells. We suggest that uPAR, because it is a GPI-anchored protein, must be present in discrete "rafts" in the cell surface to function. Our proposal has two parts. First, we will set up systems in our lab for studying signaling through uPAR in cultured human breast cancer cells. Second, we will disrupt rafts, and determine whether signal transduction is affected. Our most important advance this year has been in developing tools for monitoring raft disruption. Agents that remove cholesterol from membranes (such as methyl-beta-cyclodextrin) or that sequester cholesterol within the membrane (such as filipin) can disrupt rafts. We have shown that these agents strongly inhibit normal raft-dependent clustering of raft proteins and lipids in the plasma membrane, and that this inhibition is easily detectable by fluorescence microscopy. This will be an important tool in later experiments to examine the effect of raft disruption on uPAR-mediated signaling and cell motility. We anticipate in the next year, we will develop improved methods for detecting uPAR in rafts in cells. We will then determine how the localization of uPAR in rafts governs its deadly activity in metastasis.

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INTRODUCTION

uPAR, the urokinase-type plasminogen activator receptor, is a key player in metastasis of breast cancer cells. Our hypothesis is that uPAR must be present in discrete subregions or "rafts" in the cell surface to function. The rationale for this hypothesis is that uPAR is a member of a distinctive class of cell-surface proteins, called GPI-anchored proteins. All these proteins are anchored in the plasma membrane that surrounds each cell by attachment to a lipid molecule, instead of by having a portion of the protein itself pass through the plasma membrane as is true for most proteins on the cell surface. This lipid anchor targets any protein to which it is attached to rafts in the membrane. Work on different members of this class of lipid-anchored proteins has shown that several of them can participate in signal transduction cascades, and in several cases (especially cells of the immune system), these proteins must be present in rafts in order to send signals. Because uPAR is also lipid-anchored, we hypothesize that it must also be in rafts to send signals. Our proposal has two parts. First, we will set up systems in our lab for studying signaling through uPAR in cultured human breast cancer cells. Second, we will disrupt rafts, and determine whether signal transduction is affected. Rafts will be disrupted by three different means, in each case altering the lipid composition of the membrane. All three methods are based on knowledge of how lipids are organized in rafts. We found that lipids in rafts are packed tightly together. Agents that counteract this tight packing disrupt rafts, and may thus inhibit uPAR signaling. In this first year of the award, we have initiated studies to characterize lipid rafts, the association of GPI-anchored proteins with them, and how rafts can be disrupted.

BODY

Task 1. To establish systems for uPAR signaling in breast cancer cells in our lab, and to determine whether uPAR functions observed in other cells are also seen in breast cancer cells. Progress on this Task has been disappointingly slow. An unexpected technical hurdle has been our inability to detect uPAR cleanly using commercially available antibodies. We started by trying to detect uPAR in MCF7 breast cancer cells by standard immunofluorescence microscopy, using commercially available antibodies (American Diagnostica, Inc). We detected only a very dim, background-like staining. Increasing the concentration of primary or secondary antibodies did not improve specific staining, although non-specific background staining (of similar intensity with or without primary antibodies) was observed as the concentration of secondary antibodies was increased. In an attempt to improve weak staining, we tried secondary antibodies linked to a variety of fluorophores. These included fluorophores of the Alexa series from Molecular Probes, often considered to be more intense than the conventional fluorescein, rhodamine, or Texas red stains. We also attempted to enhance staining using a "sandwich" technique, adding an additional layer of antibodies to amplify the signal. None of these approaches were successful.

It seemed possible that MCF7 cells might contain relatively low levels of uPAR. If so, and if the antibody was sub-optimal, better results might be obtained with different cells. For this reason, we also examined 3 other breast cancer cell lines; MCA-MB-231 (reported to express high levels of uPAR), MDA-MB-435, and SKBR3. We were unable to obtain unambiguous results on any of these lines. Finally, hoping to obtain positive results even if it were not in breast cancer cells, we examined primary HUVEC (human umbilical vein endothelial cells), obtained in collaboration with Dr. Martha Furie (University at Stony Brook, Stony Brook NY), expected to be a rich source of uPAR. Although we detected a dim cell-surface stain in these cells, it was not sufficient for further

analysis. As we routinely perform immunofluorescence microscopy, detecting other proteins in other cell types, in the laboratory, our methodology and appears to be adequate for this technique.

We also attempted to detect uPAR by Western blotting, using the same antibodies. All of the cell and tissue sources described above were examined. We were unable to unambiguously detect the ca. 50 kDa uPAR protein, above the considerable background, from any of these. Various conditions of antibody concentration, blocking, and sample pre-treatment were without effect. Similar results were obtained using nitrocellulose or nylon membranes. Progress on this Task has been further delayed by the unexpected departure of the post-doctoral fellow who had been performing these studies. However, a new fellow will join the lab shortly, and will resume the project. We have recently become aware that other investigators have reported similar difficulties using the commercially available antibodies to detect uPAR. Several investigators have generated antibodies in their labs for this purpose. As detecting uPAR is crucial for the experiments proposed in Task 1 and also in Task 3, we are currently the possibility of obtaining antibodies from other labs for these studies.

Task 2. Disrupt cholesterol and sphingolipid-rich rafts. We have made the most progress on the last part of Task 2, determining the effect of raft disruption on the cell-surface distribution of raft components (GPI-anchored proteins and the raft lipid GM1) by fluorescence microscopy. Recent work has shown that rafts are much smaller than originally thought, often below the limits of detection of light microscopy. Thus, raft markers often appear to have a relatively uniform distribution on the surface of cells when examined by light microscopy. However, manipulations to experimentally bring these small rafts together in the plane of the membrane cause them to coalesce into larger structures that are easily detectable by light microscopy.

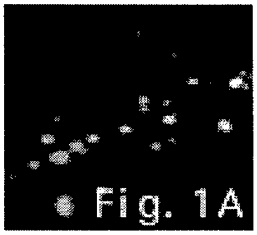
Rafts can be experimentally clustered in either of two ways. First, antibodies directed against individual raft proteins (such as GPI-anchored proteins) are added to cells. Because each antibody molecule has two identical antigen combining sites, it can bind simultaneously to two different antigen molecules. This binding causes the two antigen molecules, which had previously diffused independently in the plane of the membrane, into close proximity. Polyclonal antiserum contains a mixture of different antibody species, each of which may recognize a different individual epitope in the antigen molecule. Thus, if polyclonal antibodies are used, different antibody molecules can bind simultaneously to different epitopes on the same antigen. Combined with the bivalency of individual antibody molecules, this property can enhance antigen clustering. Secondary antibodies, directed against the primary antibodies as antigen, are generally added to enhance clustering further. Binding of these bivalent antibodies to different epitopes on the primary antibodies causes them to cluster together. The net result is extensive clustering of the original antigens in the membrane. For this reason, adding primary and secondary antibodies to the surface of cells causes the antigen molecules to collect into clusters, giving the antigen a more punctate distribution in the membrane when observed by immunofluorescence microscopy than in the absence of clustering. This difference is readily detectable by fluorescence microscopy.

The second tool for clustering rafts takes advantage of the fact that gangliosides (acidic glycosphingolipids), which are highly concentrated in rafts, can be bound by a number of bacterial toxins. Many of these toxins are targeted to rafts by ganglioside binding, and this raft targeting is essential for cell entry and intoxication (Wolf et al., 1998). A useful toxin is cholera toxin. This toxin consists of 2 subunits, A and B. The pentameric B subunit binds 5 molecules of the ganglioside GM1 or certain other related gangliosides, causing them to cluster together. Subsequent to this binding, entry of the A subunit into cells leads to toxic effects. Experimentally, the isolated

B subunit can be used to cluster gangliosides, without danger of the toxicity that would derive from the A subunit. The cholera toxin B subunit can be labeled directly with fluorescent probes such as fluorescein for detection using fluorescence microscopy. Alternatively, using the same approach as described above for antibody-mediated clustering of membrane proteins, antibodies directed against the cholera toxin B subunit can be used to further enhance clustering (Harder et al., 1998; Janes et al., 1999).

If protein antigens or gangliosides are initially present in rafts, clustering has the potential to collect the rafts together (Brown and London, 1998). It may be imagined that clustering antibodies and/or toxins "drag" small rafts along as the antigen molecules within them are clustered, and cause these small rafts to coalesce into larger rafts. Several characteristic features of the clustering behavior of raft proteins and lipids are evident. First, the clusters can be much larger than simple clustering mediated by antibody-crosslinking of the proteins. The large size of these clusters probably represents an additional level of affinity of crosslinked rafts for each other. That is, small clusters, containing raft molecules clustered by antibodies brought together by antibodies, may have an affinity for each other, and associate into larger clusters in an antibody-independent but raft-dependent manner. A second feature of these raft clusters, that distinguishes them from simple antibody-mediated clusters, is that different raft markers can inhabit the same cluster (Harder et al., 1998; Janes et al., 1999). For instance, antibodies against a particular GPI-anchored protein may be used to cause rafts to cluster. Subsequently, it can be determined that gangliosides such as GM1 are also concentrated in the same cluster. Because no toxin or other molecule that directly bound to the GM1 molecule was used, it must be concentrated in the same clusters as the GPI-anchored protein because it was in the same small raft before clustering. Direct antibody-mediated clustering of the protein caused the rafts containing the proteins to cluster, and indirectly dragged along lipids in these rafts, including GM1.

We proposed to use this system as a probe for the raft disruption techniques described in Aim 2. Results are shown for raft disruption by the polyene antibiotic filipin. Filipin inserts into membranes and then forms complexes with cholesterol. In doing so, it prevents cholesterol from engaging in the other types of lipid interaction, including those required for raft formation. Thus, treating membranes with filipin should disrupt rafts. When individual raft components are clustered with antibodies or toxins, much smaller clusters are expected. These small clusters derive only from direct association of antibody and/or toxin molecules with their membrane targets, without the additional effect of raft clustering.



We first examined the effect of filipin treatment on GM1 clustering. Cholera toxin covalently linked to the fluorescent dye fluorescein was added to the surface of cells. Cells were maintained at a low temperature (15°), to avoid endocytosis and internalization of the toxin, as this would complicate analysis. Once bound, excess toxin was washed away. Next, antibodies directed against the toxin were added. Even at reduced temperature, the diffusion rate of the lipid in the membrane was sufficient to allow clustering. After incubating for 1 hour to allow binding and clustering, excess antibodies were washed away. Cells were fixed with paraformaldehyde, and cholera toxin fluorescence was observed as shown in Fig. 1A. Large, fairly dispersed puncta are visible. The experiment was then repeated in the presence of 100 micrograms/ml of filipin. Under these conditions, much smaller, "fuzzier" puncta are seen (Fig. 1B). Similar results were obtained when rafts were disrupted with methyl-beta cyclodextrin (MBCD), which efficiently removes cholesterol from membranes (not shown). This

result clearly shows that rafts are efficiently disrupted by these agents, and that the distribution of clustered GM1, visualized microscopically, is a useful tool for monitoring raft disruption.

In Task 2, we also proposed to examine the effect of raft disruption on cell-surface distribution and clustering of the GPI-anchored protein uPAR. As described in detail below, we have had great difficulties localizing uPAR by immunofluorescence microscopy using commercially available antibodies. Alternate strategies to overcome this problem are in progress. In the meantime, it is useful to develop this methodology in the lab using another GPI-anchored protein. As different GPI-anchored proteins are expected to associate with rafts in a similar manner, working out techniques for visualizing the effect of raft disruption on a "model" GPI-anchored proteins, for which good antibodies are available, will greatly facilitate later studies on uPAR. For this reason, we examined the GPI-anchored protein placental alkaline phosphatase (PLAP). Commercially available rabbit polyclonal antibodies were added to cells maintained at reduced temperature (15°), and cells were incubated for 1 hour to allow antibody binding. Excess antibodies were washed away, and fluorescein-conjugated secondary antibodies (goat anti-rabbit IgG) were added and incubated for one hour. As described above, this procedure allowed crosslinking of the primary antibodies along with their bound ligands, further enhancing clustering. Because the secondary antibodies were linked to fluorescein, they also served as the detection probe.

Very striking large, dispersed puncta were observed (Fig. 2A). These were generally larger than the GM1 clusters shown in Fig. 1A. This may result from the fact that PLAP, as a protein, has more epitopes and can be clustered more efficiently by polyclonal antibodies than the structurally simpler GM1. Alternatively, the affinity of PLAP for rafts may be greater than GM1. (PLAP is a dimer, which would be expected to increase its raft affinity. However, the fact that cholera toxin is pentameric may argue against this possibility). It is even possible that GM1 and PLAP can inhabit different types of raft, although this possibility would require further study outside the scope of this project.

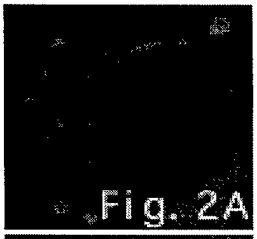


Fig. 2A

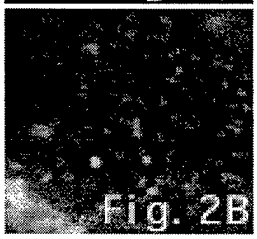


Fig. 2B

We next repeated the experiment, treating cells with 100 micrograms/ml filipin during both antibody-binding steps. Under these conditions, much smaller, dispersed puncta were observed (Fig. 2B). These were very similar to the GM1 puncta after filipin treatment. Similar results were observed when rafts were disrupted with MBCD (not shown). We conclude that both filipin and MBCD efficiently disrupt rafts and prevent raft clustering. Both GM1 and the GPI-anchored protein PLAP are useful markers of this effect. We expect that uPAR will show similar behavior.

Task 3. Apply the raft-disruption methods in Task 2 to the uPAR functional assays in Task 1. The difficulty in detecting uPAR with available antibodies, described in the section of the report on Task 1, has prevented us from starting on this task. We anticipate that we will soon overcome this difficulty using antibodies from other labs, and will be able to make good progress on this task in the upcoming months.

KEY RESEARCH ACCOMPLISHMENTS

As detailed in the Body, the most important research accomplishment has been the demonstration that the disruption of rafts using methyl-beta-cyclodextrin or filipin can be visualized

in cells by fluorescence microscopy, by detecting the striking effect on the morphology of antibody- or toxin-mediated clusters of raft components. This will be an important tool in later studies.

REPORTABLE OUTCOMES

The principle investigator gave presentations including the work described here at the following venues:

09/10/01; Dept. of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN
09/25/01; Biochemistry Dept., Boston University School of Medicine, Boston, MA
10/09/01; Integrated Graduate Program, Northwestern University School of Medicine, Chicago, IL
01/16/02; Oregon Health & Sciences University, Dept Physiology and Pharmacology, Portland, OR
02/04/01; Dept. Physiology & Biophysics, Robert Wood Johnson Medical School, Piscataway, NJ
02/28/02; Biochemistry Dept., Case Western Reserve University, Cleveland, OH
04/04/02; Biochemistry and Molecular Biology Dept., Michigan State University, E. Lansing, MI

There were no publications of other reportable outcomes of this work in the past year.

CONCLUSIONS

The medical significance of this work remains as described in the original proposal. It is clear that uPA interactions with uPAR play a key role in metastasis, the deadliest feature of breast cancer. Our findings strongly suggest that the presence of uPAR, a GPI-anchored protein, in membrane rafts affects its signaling and its ability to govern cell migration during metastasis. As methods for disrupting rafts are becoming more readily available, the importance of testing the ability of these compounds to inhibit uPAR signaling has never been greater.

REFERENCES

Brown, D.A., and London, E. 1998. Structure and origin of ordered lipid domains in biological membranes. *J. Membr. Biol.* 164: 103-114.

Harder, T., Scheiffele, P., Verkade, P., and Simons, K. 1998. Lipid-domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* 141: 929-942.

Janes, P.W., Ley, S.C., and Magee, A.I. 1999. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J. Cell Biol.* 147: 447-461.

Wolf, A.A., Jobling, M.G., Wimer-Mackin, S., Ferguson-Maltzman, M., Madara, J.L., Holmes, R.K., and Lencer, W.I. 1998. Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J. Cell Biol.* 141: 917-927.